CONCISE COMMUNICATION

Differential invasion of respiratory epithelial cells by members of the *Burkholderia cepacia* complex

P. M. Keig[1], *E. Ingham*[1], *P. A. R. Vandamme*[2] *and K. G. Kerr*[1]

[1]Division of Microbiology, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK, and [2]Laboratorium voor Microbiologie, Universiteit Gent, Ledeganckstraat 35, B−9000, Gent, Belgium

To investigate whether there are differences between members of the *Burkholderia cepacia* complex in their ability to invade human respiratory epithelial cells, 11 strains belonging to genomovars I–V were studied in an antibiotic protection assay using the A549 cell line. Strains belonging to genomovars II and III were more invasive than those of genomovars I, IV and V. There was also intra-genomovar variation in invasiveness. No correlation between invasiveness and other putative virulence factors of importance in *B. cepacia* infection in individuals with cystic fibrosis, cable pilus and *B. cepacia* epidemic strain marker was identified.

Keywords *Burkholderia cepacia*, respiratory epithelium

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Pulmonary colonization in cystic fibrosis (CF) patients with *Burkholderia cepacia* may lead to one of three clinical outcomes: persistent colonization without deterioration, accelerated reduction in lung function, or in about 20% of patients, rapid deterioration with a fatal necrotizing pneumonia sometimes accompanied by bacteremia known as the 'cepacia syndrome' [1]. It is not known what factors are responsible for these differences. Although host factors are likely to be of importance, it is also possible that they may be due, at least in part, to differences in the *B. cepacia* strains infecting CF patients.

Each of the six genomovars which comprise the *B. cepacia* complex have been recovered from CF patients, however, strains of genomovars II (*B. multivorans*) and III account for the majority of CF isolates. The latter represent the majority of epidemic strains of *B. cepacia* and are associated with greater morbidity and mortality than any other members of the *B. cepacia* complex [2,3]. However, the distribution of genomovars in the CF patient community has not been systematically determined and the pathogenic significance of the different genomovars is not known.

Virulence markers such as the cable (cbl) pilus [4] and the *B. cepacia* epidemic strain marker (BCESM) [5] occur almost exclusively in genomovar III. However, not all outbreak strains belong to genomovar III. Moreover, the BCESM is also found outside genomovar III [6]. Comparison of the natural history of colonization by genomovar II and III strains has revealed striking differences and it has been suggested that genomovar III colonized patients are more likely to experience an adverse clinical outcome [2]. This evidence suggests that there are differences in the pathogenic potential of strains belonging to different genomovars.

Despite the acknowledged significance of *B. cepacia*, comparatively little is known of the virulence factors of importance in the natural history of infection associated with the bacterium. There is, however, convincing evidence to suggest that *B. cepacia* can invade and survive within human cells [7–9]. The aim of this study, therefore, was to determine whether there are differences between members of the *B. cepacia* complex in their ability to invade respiratory epithelial cells.

The *B. cepacia* strains used, all from the BCCM/LMG Bacteria Collection, University of Gent,

Corresponding author and reprint requests: Dr Kevin G. Kerr, Division of Microbiology, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK
Tel: +44 113233 5617
Fax: +44 113233 5669
E-mail: mickgk@leeds.ac.uk

Table 1 Bacterial strains

Strain	Genomovar	Source
LMG 1222	I	Onion
LMG 18821	I	CF patient (Australia)
LMG 13010	II	CF patient (Belgium)
LMG 17588	II	Soil (USA)
LMG12615	III	CF patient (UK)
LMG 16659	III	CF patient (UK)
LMG 14271	III	CF patient (Belgium)
LMG 7000	IV	Blood culture (Sweden)
LMG 14291	IV	CF patient (Belgium)
LMG 10929	V	Rice rhizosphere (Vietnam)
LMG 16232	V	CF patient (Sweden)

CF, cystic fibrosis.

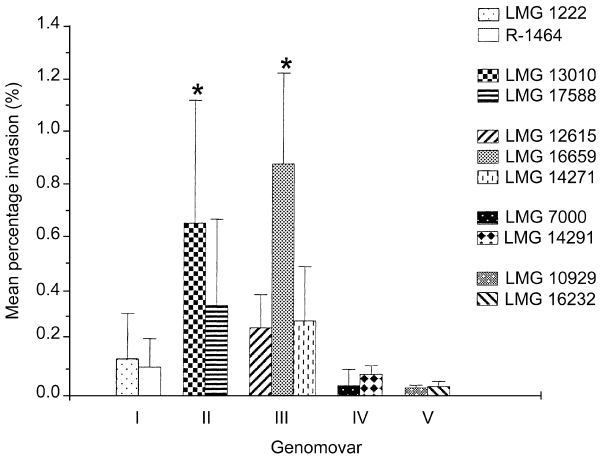

Figure 1 Invasion of A549 respiratory epithelial cells by members of the *Burkholderia cepacia* complex. Results are expressed as mean of duplicate wells of three independent experiments ($n = 6$) ± 95% confidence limits. Asterisks indicate invasion significantly different from all other strains ($P < 0.05$).

Belgium, are shown in Table 1. Invasiveness was determined in a ceftazidime–gentamicin protection assay, as described previously [8], by laboratory workers blinded to the identity of each isolate. Briefly, monolayers of A549 cells were prepared in 24-well tissue culture plates and were infected with a suspension of stationary-phase (approximately 16 h) bacteria to yield a multiplicity of infection of approximately 10 bacteria per A549 cell. Stationary-phase bacteria were used in preference to cells in the exponential phase, as invasiveness is greater under the former conditions [10]. The numbers of bacteria added were determined by viable cell counts on Iso-Sensitest agar (Unipath Ltd., Basingstoke, UK). Infected monolayers were then incubated at 37 °C in a humid atmosphere containing 5% CO_2 for 2 h, whereupon they were washed thrice with sterile phosphate-buffered saline. Low protein hybridoma medium (LPHM, Gibco, Paisley, UK) containing 500 mg/L ceftazidime, and 160 mg/L gentamicin was then added to prevent further growth of extracellular bacteria. This was taken as time-point zero (T0). Numbers of intracellular bacteria [colony-forming units (CFU)/mL] were determined by viable cell counts of serial dilutions on Iso-Sensitest agar following lysis of the pneumocytes with 0.3% (v/v) Triton X-100 (Sigma, Poole, UK) at T0 and T2 post-infection. Experiments were performed in duplicate on three separate occasions. The number of bacteria at T0 was assumed to represent the number of internal bacteria plus the number of extracellular bacteria (i.e. bacteria attached to the monolayer), whereas numbers of bacteria at T2 post-infection were taken to represent only intracellular bacteria. Assays for each strain were performed in duplicate on three separate occasions

Results were analysed by either one-way or two-way analyses of variance (ANOVA). Minimum significant differences (MSD) between means ($n = 6$) were calculated by the T method [11].

All the strains of members of the *B. cepacia* complex examined in this study were found to invade and survive within respiratory epithelial cells (Figure 1). However, differences in the degree of invasiveness between genomovars were observed. Strains belonging to genomovars II and III were found to be more invasive in contrast to the strains of genomovars I, IV and V. This, along with the observation that most CF isolates belong to genomovars II and III, and that genomovar III isolates comprise the epidemic strains associated with greater morbidity and mortality [2,3], implies that CF patients colonized with genomovar II and III isolates might possibly be at a greater risk of an adverse clinical outcome following infection with these genomovars.

Significant differences in invasion between strains within each of the genomovars II and III were also found. The genomovar II CF isolate was significantly more invasive than the soil isolate, although the latter was still more invasive than any of the strains belonging to genomovars I, IV, or V. In addition, it did not differ significantly in invasiveness to strains LMG 12615 and LMG 14271 of genomovar III. Therefore, environmental as well as clinical strains of *B. cepacia* of genomovar II might also present a potential risk to CF patients.

Within genomovar III, the BCESM$^+$ epidemic strain LMG 16659 associated with a poor clinical outcome was significantly more invasive compared with the other two genomovar III strains; LMG 12615 (an epidemic strain of the ET12 lineage possessing the cbl pilus and the BCESM marker); and LMG 14271, a cbl$^-$ BCESM$^-$ strain, which were not associated with a bad outcome. There was, however, no significant difference in invasiveness between the latter. The degree of invasiveness appears therefore to have no correlation with the presence or absence of either the cbl pilus or the BCESM marker.

The limitations of this study must be acknowledged — only a very small number of examples of each genomovar were studied. Nevertheless, evidence from this preliminary study suggests that there are differences in invasiveness between members of the *B. cepacia* complex, with strains belonging to genomovars II and III being more invasive than strains belonging to genomovars I, IV and V and that the invasive phenotype may be associated with an adverse clinical outcome independently of cbl pilus and BCESM. Further studies are needed to determine the validity of the associations between clinical outcome, genomovar and invasiveness for respiratory epithelial cells, as this may yield not only important prognostic information, but may also permit relaxation of the stringent isolation policies employed by CF centres when patients are identified as being colonized by *B. cepacia*.

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